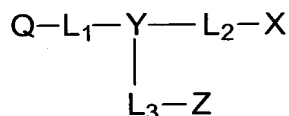


## CLAIMS

We claim:

A fluorescence quencher composition having the structure:



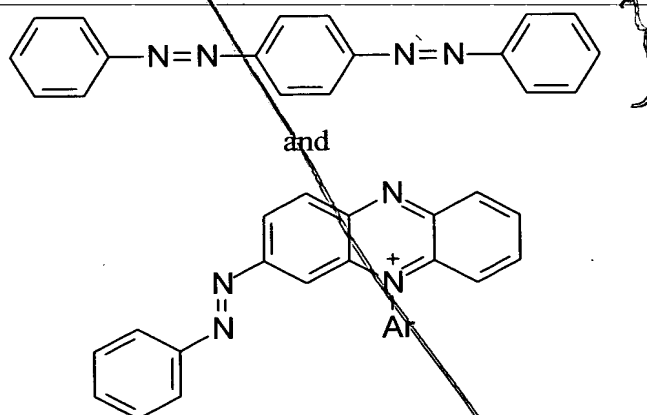
5 wherein Y is selected from N and CR, where R is H, C<sub>1</sub>-C<sub>6</sub> alkyl or C<sub>5</sub>-C<sub>14</sub> aryl;

L<sub>1</sub>, L<sub>2</sub>, and L<sub>3</sub> are independently selected from a bond, C<sub>1</sub>-C<sub>12</sub> alkylidyl, C<sub>1</sub>-C<sub>12</sub> alkoxyldyl, C<sub>1</sub>-C<sub>12</sub> alkylaminodiy, C<sub>1</sub>-C<sub>12</sub> alkylamidediyl, C<sub>5</sub>-C<sub>14</sub> aryldiy, and 1-20 ethyleneoxy units;

10 X is an amino acid, a polypeptide, a nucleoside, a nucleotide, a polynucleotide, or a protected form thereof; or X is an acid-labile protecting group;

Z is selected from H, CO<sub>2</sub>H, OH, NH<sub>2</sub>, NHR, NR<sub>2</sub>, SH, an ester, a cleavable linker, a solid support, a reactive linking group, and a label selected from a fluorescent dye, a hybridization-stabilizing moiety, a chemiluminescent dye, and an affinity ligand; and

Q is selected from the diazo structures:



20 wherein Ar is C<sub>5</sub>-C<sub>14</sub> aryl; one of the aryl carbons of the diazo structures is the site of attachment to L<sub>1</sub>; at least one aryl carbon of each diazo structure is substituted with an electron-withdrawing group and at least one aryl carbon of each diazo structure is substituted with an electron-donating group.

2. The fluorescence quencher composition of claim 1 wherein the electron-withdrawing groups are selected from NO<sub>2</sub>, CN, CF<sub>3</sub>, CO<sub>2</sub>H, CO<sub>2</sub>R, C(O)NH<sub>2</sub>, C(O)NHR, C(O)NR<sub>2</sub>, CHO, C(O)R, SO<sub>2</sub>R, SO<sub>2</sub>CF<sub>3</sub>, SO<sub>2</sub>OR, SO<sub>3</sub>H, NO, and C<sub>5</sub>-C<sub>14</sub> aryl, where R is H, C<sub>1</sub>-C<sub>12</sub> alkyl or C<sub>5</sub>-C<sub>14</sub> aryl.

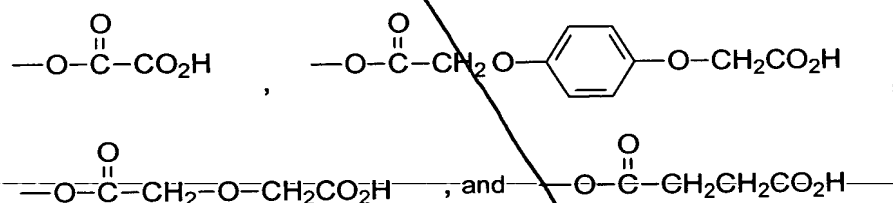
3. The fluorescence quencher composition of claim 2 wherein a NO<sub>2</sub> is *para* to a diazo group.

4. The fluorescence quencher composition of claim 1 wherein the electron-donating groups are selected from O<sup>-</sup>, S<sup>-</sup>, NR<sub>2</sub>, NHR, NH<sub>2</sub>, NHC(O)R, OR, OH, OC(O)R, SR, SH, Br, I, Cl, F, R, and C<sub>5</sub>-C<sub>14</sub> aryl, where R is H, C<sub>1</sub>-C<sub>12</sub> alkyl or C<sub>5</sub>-C<sub>14</sub> aryl.

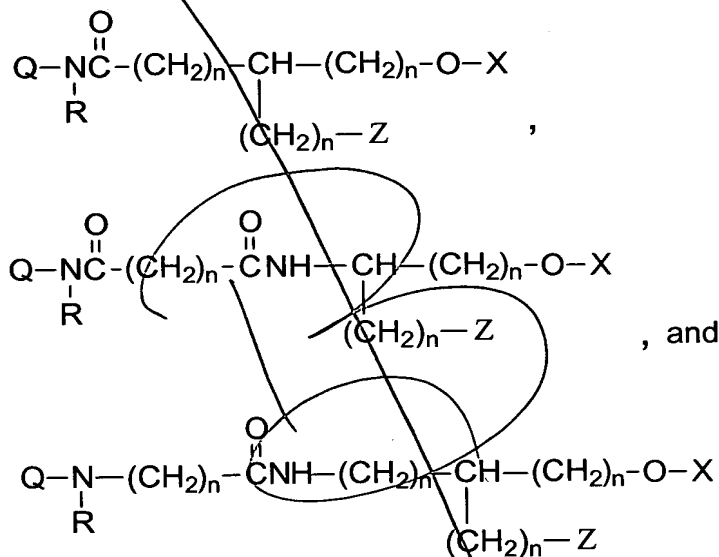
5. The fluorescence quencher composition of claim 4 wherein a OCH<sub>3</sub> is *ortho* or *meta* to a diazo group.

6. The fluorescence quencher composition of claim 1 where Z is OH

7. The fluorescence quencher composition of claim 1 where Z is an ester selected from the structures:



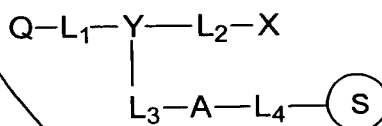
8. The fluorescence quencher composition of claim 1 selected from the structures:



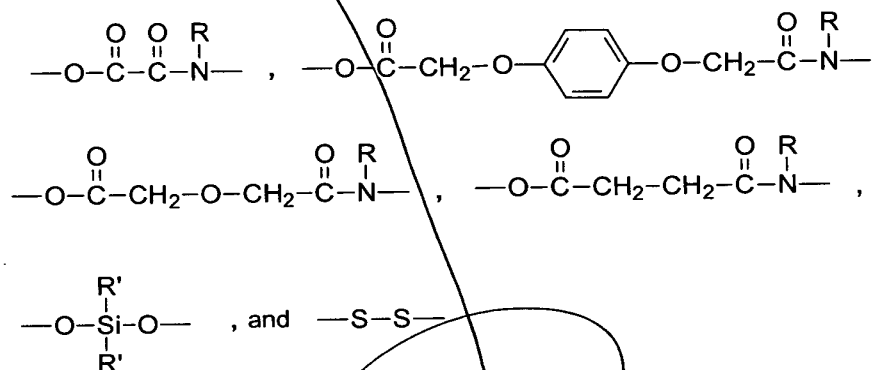
where n is 1 to 12.

9. The fluorescence quencher composition of claim 1 wherein X is selected from DMT, MMT, trityl, substituted trityl, pixyl, and trialkylsilyl.

10. The fluorescence quencher composition of claim 1 having the structure:



wherein A is a cleavable linker selected from the structures:



where R' is H, C<sub>1</sub>-C<sub>12</sub> alkyl or C<sub>1</sub>-C<sub>12</sub> alkoxy;

L<sub>4</sub> is selected from a bond, C<sub>1</sub>-C<sub>12</sub> alkylidiyl, C<sub>1</sub>-C<sub>12</sub> alkoxyldiyl, C<sub>1</sub>-C<sub>12</sub> alkylaminodiyl, C<sub>1</sub>-C<sub>12</sub> alkylamidediyl, C<sub>5</sub>-C<sub>14</sub> aryldiyl, and 1-20 ethyleneoxy units; and

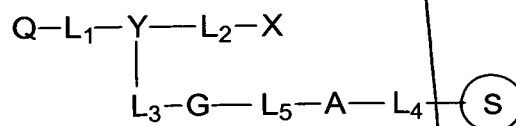
$\text{S}$  is a solid support.

11. The fluorescence quencher composition of claim 10 wherein X is a nucleotide.

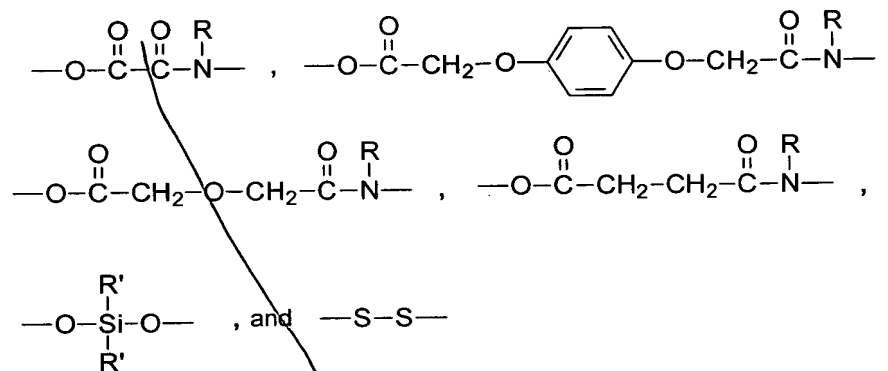
12. The fluorescence quencher composition of claim 10 wherein the solid support is selected from polystyrene, controlled-pore-glass, silica gel, silica, polyacrylamide, polyacrylate, hydroxyethylmethacrylate, polyamide, polyethylene, polyethyleneoxy, and copolymers and grafts of such.

13. The fluorescence quencher composition of claim 10 wherein the form of the solid support is selected from a particle, a bead, a membrane, a frit, a fiber, a tube, a capillary, a slide, a plate, a micromachined chip, an alkanethiol-gold layer, a magnetic bead, a non-porous surface, an addressable array, and polynucleotide-immobilizing medium.

14. The fluorescence quencher composition of claim 1 having the structure:



wherein A is a cleavable linker selected from the structures:



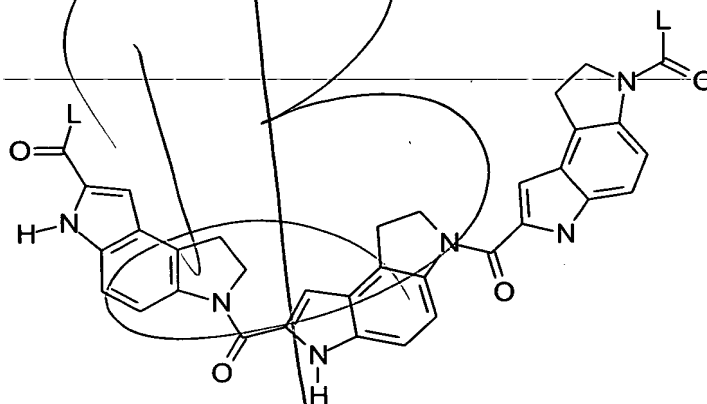
where R is H, C<sub>1</sub>–C<sub>12</sub> alkyl or C<sub>1</sub>–C<sub>12</sub> alkoxy;

L<sub>4</sub> and L<sub>5</sub> are independently selected from a bond, C<sub>1</sub>–C<sub>12</sub> alkylidyl, C<sub>1</sub>–C<sub>12</sub> alkoxyldyl, C<sub>1</sub>–C<sub>12</sub> alkylaminodiy, C<sub>1</sub>–C<sub>12</sub> alkylamididyl, C<sub>5</sub>–C<sub>14</sub> arylidyl, and 1-20 ethyleneoxy units;

G is a hybridization-stabilizing moiety; and

(S) is a solid support.

15. The fluorescence quencher composition of claim 14 in which G comprises:

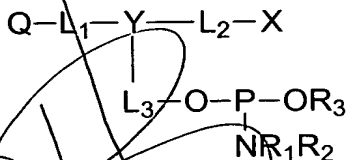


where L are the sites of attachment to L<sub>3</sub> and L<sub>5</sub>.

10      16. The fluorescence quencher composition of claim 14 wherein the solid support is selected from polystyrene, controlled-pore-glass, silica gel, silica, polyacrylamide, magnetic beads, polyacrylate, hydroxyethylmethacrylate, polyamide, polyethylene, polyethyleneoxy, and copolymers and grafts of such.

15      17. The fluorescence quencher composition of claim 14 wherein the form of the solid support is selected from a particle, a bead, a membrane, a frit, a fiber, a tube, a capillary, a slide, a plate, a micromachined chip, an alkanethiol-gold layer, a magnetic bead, a non-porous surface, an addressable array, and polynucleotide-immobilizing medium.

18. The fluorescence quencher composition of claim 1 having the structure:

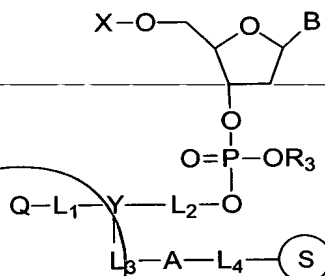


wherein X is an acid-labile protecting group; R<sub>1</sub> and R<sub>2</sub> are individually selected from isopropyl, morpholino, methyl, ethyl and C<sub>5</sub>-C<sub>14</sub> aryl; R<sub>1</sub> and R<sub>2</sub> taken together are C<sub>4</sub>-C<sub>11</sub> cycloalkyl or morpholino; and R<sub>3</sub> is C<sub>1</sub>-C<sub>6</sub> alkyl or C<sub>5</sub>-C<sub>14</sub> aryl.

19. The fluorescence quencher composition of claim 18 wherein R<sub>1</sub> and R<sub>2</sub> are each isopropyl and R<sub>3</sub> is cyanoethyl.

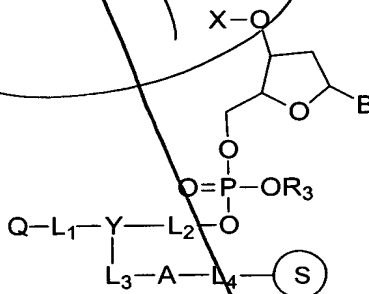
20. The fluorescence quencher composition of claim 18 wherein X is selected from DMT, MMT, trityl, substituted trityl, pixyl, and trialkylsilyl.

21. The fluorescence quencher composition of claim 11 having the structure:



wherein X is an acid-labile protecting group; B is a nucleobase; and R<sub>3</sub> is selected from H, C<sub>1</sub>-C<sub>6</sub> alkyl, and C<sub>5</sub>-C<sub>14</sub> aryl.

22. The fluorescence quencher composition of claim 11 having the structure:



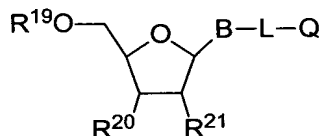
wherein X is an acid-labile protecting group; B is a nucleobase; and R<sub>3</sub> is selected from H, C<sub>1</sub>-C<sub>6</sub> alkyl, and C<sub>5</sub>-C<sub>14</sub> aryl.

23. The fluorescence quencher composition of claim 1 where X is a polynucleotide.

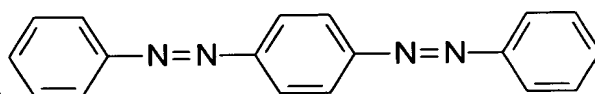
24. The fluorescence quencher composition of claim 23 wherein the polynucleotide comprises one or more N-[2-(aminoethyl)]glycine units having a nucleobase attached to nitrogen through a methylene carbonyl linkage.

25. The fluorescence quencher composition of claim 23 wherein the polynucleotide comprises one or more 2'-4' or 3'-4' bicyclic sugar modifications.

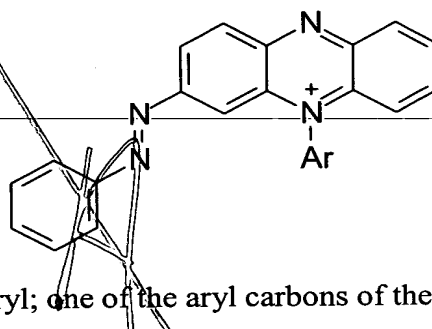
26. A labelled nucleoside or nucleotide having the formula:



wherein Q is a quencher moiety selected from the diazo structures:



and



wherein Ar is C<sub>5</sub>-C<sub>14</sub> aryl; one of the aryl carbons of the diazo structures is the site of attachment to L; at least one aryl carbon of each diazo structure is substituted with an electron-withdrawing group and at least one aryl carbon of each diazo structure is substituted with an electron-donating group;

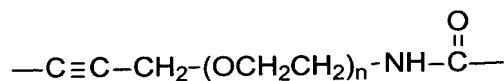
B is a nucleobase;

R<sup>19</sup> is H, monophosphate, diphosphate, triphosphate, thiophosphate, phosphate analog, or acid-labile protecting group;

R<sup>20</sup> and R<sup>21</sup>, when taken alone, are each independently H, HO, F, or a moiety which terminates polymerase-mediated target-directed polymerization; or when taken together form 2'-3'-didehydroribose; and

L is a linker comprising an alkynyl, propargyl, propargylethoxyamido, vinyl, or allyl group.

27. The labelled nucleoside or nucleotide of claim 26 in which L comprises:



wherein n is 0, 1, or 2.

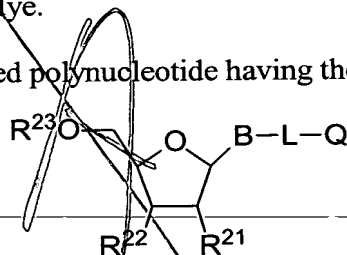
28. The labelled nucleoside or nucleotide of claim 26 which is enzymatically incorporatable.

29. The labelled nucleoside or nucleotide of claim 26 which is enzymatically extendable.

30. The labelled nucleoside or nucleotide of claim 26 which is a terminator.

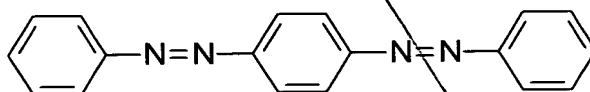
31. The labelled nucleoside or nucleotide of claim 26 wherein Q further comprises a fluorescent dye, wherein the fluorescent dye and quencher moiety are covalently attached by a linker; and the fluorescent dye is selected from a fluorescein dye, a rhodamine dye, a benzophenoxazine, and a cyanine dye.

32. A nucleobase-labelled polynucleotide having the formula:

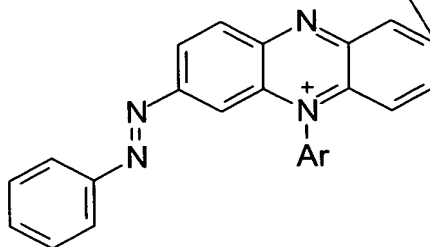


comprising 2 to 100 nucleotides, wherein

Q is a quencher moiety selected from the diazo structures:



and



wherein Ar is C<sub>5</sub>-C<sub>14</sub> aryl; one of the aryl carbons of the diazo structures is the site of attachment to L; at least one aryl carbon of each diazo structure is substituted with an electron-withdrawing group and at least one aryl carbon of each diazo structure is substituted with an electron-donating group;

B is a nucleobase;

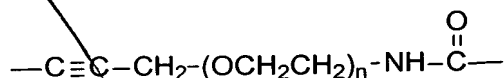
$R^{21}$  is H, OH, halide, azide, amine,  $C_1-C_6$  aminoalkyl,  $C_1-C_6$  alkyl, allyl,  $C_1-C_6$  alkoxy,  $-OCH_3$ , or  $-OCH_2CH=CH_2$ ;

$R^{22}$  is H, phosphate, internucleotide phosphodiester, or internucleotide analog;

$R^{23}$  is H, phosphate, internucleotide phosphodiester, or internucleotide analog; and

5 L is a linker comprising an alkynyl, propargyl, propargylethoxyamido, vinyl, allyl, or  $C_1-C_{12}$  alkylidyl group.

33. The nucleobase-labelled polynucleotide of claim 32 in which L comprises:



wherein n is 0, 1, or 2.

10 34. The nucleobase-labelled polynucleotide of claim 32 which further comprises one or more N-[2-(aminoethyl)]glycine units having a nucleobase attached to nitrogen through a methylene carbonyl linkage.

35. The nucleobase-labelled polynucleotide of claim 32 which further comprises one or more 2'-4' or 3'-4' bicyclic sugar modifications.

15 36. The nucleobase-labelled polynucleotide of claim 32 wherein Q further comprises a fluorescent dye, wherein the fluorescent dye and quencher moiety are covalently attached by a linker; and the fluorescent dye is selected from a fluorescein dye, a rhodamine dye, a benzophenoxazine, and a cyanine dye.

20 37. A method of labelling a polypeptide comprising the step of reacting a linking moiety of a fluorescence quencher with a polypeptide to form a labelled quencher-polypeptide conjugate,

wherein the linking moiety is selected from the group consisting of an azido, a monosubstituted primary amine, a disubstituted secondary amine, a thiol, an hydroxyl, a halide, an epoxide, an N-hydroxysuccinimidyl ester, a carboxyl, and an activated ester;

25 whereby the quencher moiety is attached to a location of the polypeptide selected from the amino terminus, the carboxyl terminus, and an amino acid side-chain.

38. A method of polynucleotide labelling comprising:

a) providing the fluorescence quencher composition of claim 1 wherein Z is a solid support,  $L_2$  is  $C_1-C_{12}$  alkoxydiyl, and X is an acid-labile protecting group;

30 b) reacting the labelled solid-support with acid to remove X;



- c) adding a 3'-phosphoramidite, 5' protected nucleoside and an activator, thereby forming a bond between L<sub>2</sub> and the 3' terminus of the nucleoside;  
d) adding an oxidizing reagent; and  
e) repeating steps b) to d) until a labelled polynucleotide is synthesized.

5 39. The method of polynucleotide labelling of claim 38 further comprising capping any unreacted sites on the solid-support after step c).

40. The method of polynucleotide labelling of claim 38 wherein the 5' terminus is attached to a fluorescent dye by a linkage, wherein the fluorescent is selected from a fluorescein, a rhodamine, a benzophenoxazine, and a cyanine.

10 41. The method of polynucleotide labelling of claim 38 wherein a nucleobase of the polynucleotide is labelled with a fluorescent dye, selected from a fluorescein, a rhodamine, a benzophenoxazine, and a cyanine, by a linkage at a position on the polynucleotide selected from the 8-position of a purine nucleobase, the 7- or 8-position of a 7-deazapurine nucleobase, and the 5-position of a pyrimidine nucleobase.

15 42. The method of polynucleotide labelling of claim 38 further comprising deprotecting the labelled polynucleotide.

20 43. The method of polynucleotide labelling of claim 38 wherein the solid-support is selected from polystyrene, controlled-pore-glass, silica gel, silica, polyacrylamide, magnetic beads, polyacrylate, hydroxyethylmethacrylate, polyamide, polyethylene, polyethyleneoxy, and copolymers and grafts of such.

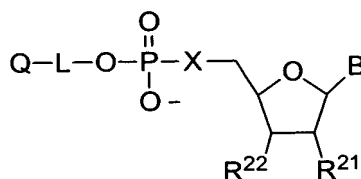
44. The method of polynucleotide labelling of claim 38 wherein the form of the solid support is selected from a particle, a bead, a membrane, a frit, a fiber, a tube, a capillary, a slide, a plate, a micromachined chip, an alkanethiol-gold layer, a magnetic bead, a non-porous surface, an addressable array, and polynucleotide-immobilizing medium.

25 45. The method of polynucleotide labelling of claim 38 wherein a plurality of polynucleotides covalently attached to a solid support in an addressable array are synthesized.

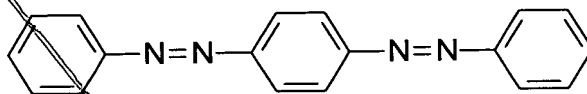
46. A method of polynucleotide labelling comprising coupling a polynucleotide with the fluorescence quencher composition of claim 18, whereby a 5' quencher labelled polynucleotide is formed.

30 47. The method of polynucleotide labelling of claim 46 wherein the 3' terminus of the polynucleotide is covalently attached to a solid support.

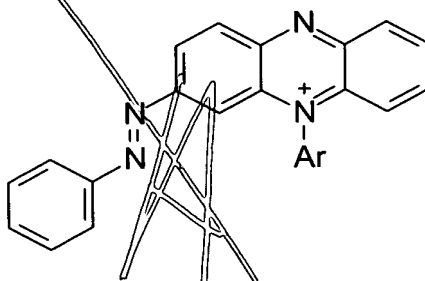
48. A 5' quencher labelled polynucleotide having the formula:



comprising 2 to 100 nucleotides, wherein Q is a quencher moiety selected from the diazo structures:



and



wherein Ar is C<sub>5</sub>-C<sub>14</sub> aryl; one of the aryl carbons of the diazo structures is the site of attachment to L; at least one aryl carbon of each diazo structure is substituted with an electron-withdrawing group and at least one aryl carbon of each diazo structure is substituted with an electron-donating group;

B is a nucleobase;

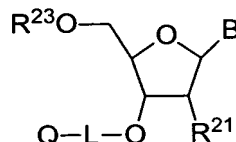
X is O, NH, or S;

R<sup>21</sup> is H, OH, halide, azide, amine, C<sub>1</sub>-C<sub>6</sub> aminoalkyl, C<sub>1</sub>-C<sub>6</sub> alkyl, allyl, C<sub>1</sub>-C<sub>6</sub> alkoxy, -OCH<sub>3</sub>, or -OCH<sub>2</sub>CH=CH<sub>2</sub>;

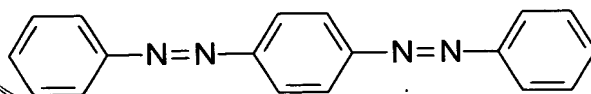
R<sup>22</sup> is internucleotide phosphodiester or internucleotide analog; and

L is C<sub>1</sub>-C<sub>12</sub> alkylidyl, arylidyl, or polyethyleneoxy.

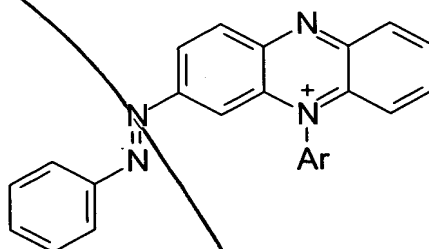
49. A 3' quencher labelled polynucleotide having the formula:



comprising 2 to 100 nucleotides, wherein Q is a quencher moiety selected from the diazo structures:



and



wherein Ar is C<sub>5</sub>-C<sub>14</sub> aryl; one of the aryl carbons of the diazo structures is the site of attachment to L; at least one aryl carbon of each diazo structure is substituted with an electron-withdrawing group and at least one aryl carbon of each diazo structure is substituted with an electron-donating group;

B is a nucleobase;

X is O, NH, or S;

R<sup>21</sup> is H, OH, halide, azide, amine, C<sub>1</sub>-C<sub>6</sub> aminoalkyl, C<sub>1</sub>-C<sub>6</sub> alkyl, allyl, C<sub>1</sub>-C<sub>6</sub> alkoxy, -OCH<sub>3</sub>, or -OCH<sub>2</sub>CH=CH<sub>2</sub>;

R<sup>23</sup> is internucleotide phosphodiester or internucleotide analog; and

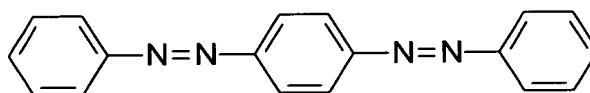
L is C<sub>1</sub>-C<sub>12</sub> alkylidyl, arylidyl, or polyethyleneoxy.

50. A method of primer extension comprising:

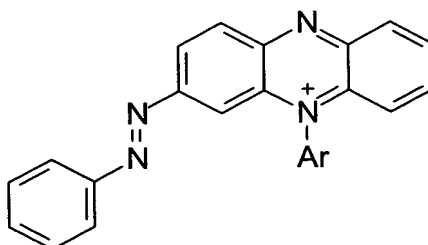
annealing a polynucleotide primer to a target polynucleotide; and

extending the primer by polymerase-mediated incorporation of a 2'-deoxynucleotide 5'-triphosphate;

wherein the primer or the nucleotide 5'-triphosphate is covalently attached by a linkage to an aryl carbon of a quencher moiety selected from the diazo structures:



and



wherein Ar is C<sub>5</sub>-C<sub>14</sub> aryl; at least one aryl carbon of each diazo structure is substituted with an electron-withdrawing group and at least one aryl carbon of each diazo structure is substituted with an electron-donating group;

whereby a labeled polynucleotide is formed.

5 51. The method of claim 50 further comprising amplifying the target polynucleotide with nucleotide 5'-triphosphates, a polymerase, and two or more primers; wherein the primers are complementary to the target polynucleotide sequence and at least one primer is covalently attached by a linkage to an aryl carbon of a quencher moiety.

10 52. The method of claim 50 further comprising amplifying the target polynucleotide with nucleotide 5'-triphosphates, a polymerase, and two or more primers; wherein the primers are complementary to the target polynucleotide sequence and at least one nucleotide 5'-triphosphate is covalently attached by a linkage to an aryl carbon of a quencher moiety.

15 53. The method of claim 50 further comprising amplifying the target polynucleotide with nucleotide 5'-triphosphates, a polymerase, two or more primers; wherein the primers are complementary to the target polynucleotide sequence, and a detectable probe; wherein the detectable probe is complementary to the target polynucleotide and is covalently attached to a fluorescent dye and a quencher moiety.

20 54. The method of claim 53 further comprising detecting a signal from the fluorescent dye of said detectable probe.

55. The method of claim 54 wherein the signal is detected at each thermal cycle during amplification.

56. The method of claim 53 wherein said polymerase cleaves the detectable probe during amplification; whereby the fluorescent dye and the quencher moiety are separated.

25 57. The method of claim 56 further comprising detecting a signal from the fluorescent dye of said cleaved, detectable probe.

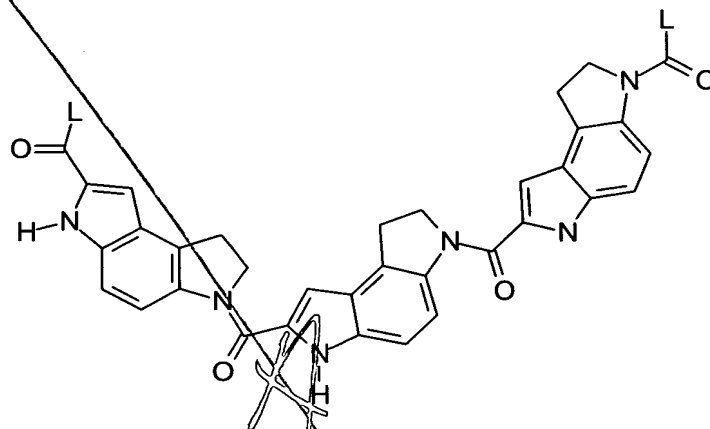
58. The method of claim 57 wherein the signal is detected at each thermal cycle during amplification.

59. The method of claim 53 wherein said fluorescent dye is attached to the 5' terminus or 3' terminus of the detectable probe.

60. The method of claim 53 wherein said quencher moiety is attached to the 5' terminus or 3' terminus of the detectable probe.

61. The method of claim 53 wherein the detectable probe is further labelled with a hybridization-stabilizing moiety.

62. The method of claim 61 wherein the hybridization-stabilizing moiety comprises the structure:



where L is an attachment site to the detectable probe.

63. The method of claim 50 further comprising:  
forming one or more labeled polynucleotide fragments by polymerase-directed primer extension of a primer;  
resolving the one or more labeled polynucleotide fragments; and  
detecting the resolved labeled polynucleotide fragments.

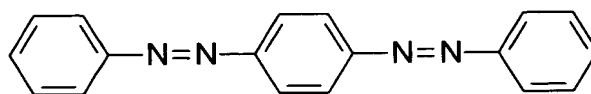
64. The method of claim 63 wherein the resolving step is an electrophoretic size-dependent separation process and the one or more labeled polynucleotide fragments are detected by fluorescence.

65. The method of claim 64 wherein the primer is covalently attached by a linkage to an aryl carbon of a quencher moiety.

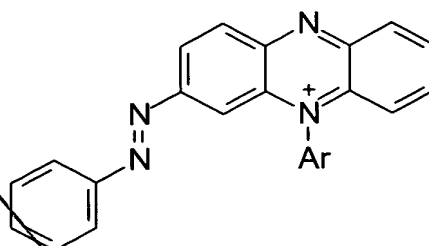
66. The method of claim 64 wherein a nucleotide 5'-triphosphate is covalently attached by a linkage to an aryl carbon of a quencher moiety.

67. A method of oligonucleotide ligation comprising annealing two probes to a target sequence and forming a phosphodiester bond with a ligase enzyme between the 5' terminus of one probe and the 3' terminus of the other probe; wherein one probe is covalently attached to a

fluorescent dye and the other probe is covalently attached by a linkage to an aryl carbon of a quencher moiety selected from the diazo structures:



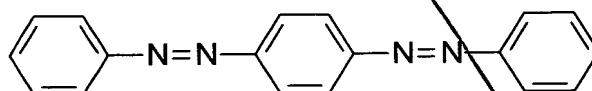
and



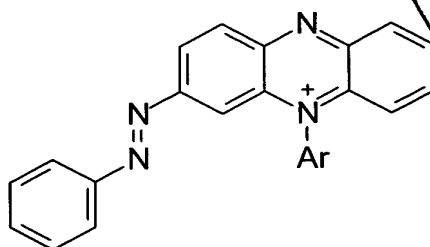
wherein Ar is C<sub>5</sub>-C<sub>14</sub> aryl; at least one aryl carbon of each diazo structure is substituted with an electron-withdrawing group and at least one aryl carbon of each diazo structure is substituted with an electron-donating group;

whereby an oligonucleotide ligation product is formed.

68. A method of hybridization detection comprising annealing a probe to a target polynucleotide sequence, wherein the probe is covalently attached to a fluorescent dye and a quencher moiety selected from the diazo structures:



and



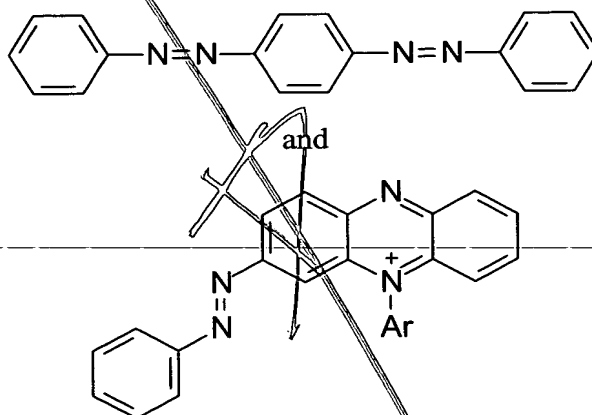
wherein Ar is C<sub>5</sub>-C<sub>14</sub> aryl; at least one aryl carbon of each diazo structure is substituted with an electron-withdrawing group and at least one aryl carbon of each diazo structure is substituted with an electron-donating group; and detecting a signal from the fluorescent dye.

69. The method of hybridization detection of claim 68 wherein the probe comprises one or more N-[2-(aminoethyl)]glycine units having a nucleobase attached to nitrogen through a methylene carbonyl linkage.

70. The method of hybridization detection of claim 68 wherein the probe comprises one or more 2'-4' or 3'-4' bicyclic sugar modifications.

71. The method of hybridization detection of claim 68 wherein the probe sequence comprises a self-complementary hairpin sequence, whereby an increase in fluorescence signal is detectable when the probe is annealed to the target sequence.

72. A kit for primer extension comprising one or more nucleotide 5'-triphosphates and one or more primers wherein at least one primer is covalently attached by a linkage to an aryl carbon of a quencher moiety selected from the diazo structures:

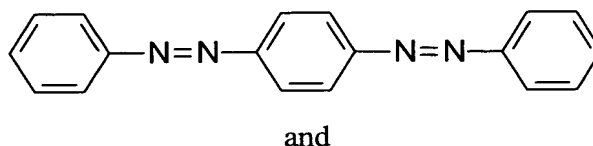


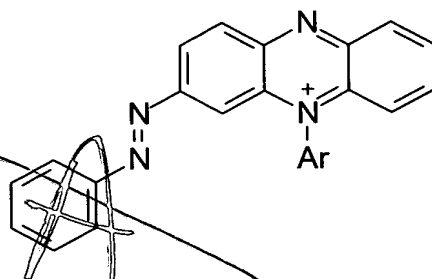
wherein Ar is C<sub>5</sub>-C<sub>14</sub> aryl; at least one aryl carbon of each diazo structure is substituted with an electron-withdrawing group and at least one aryl carbon of each diazo structure is substituted with an electron-donating group.

73. The kit of claim 72 further comprising a polymerase.

74. The kit of claim 72 further comprising a chain-terminating nucleotide analog.

75. A kit for nucleic acid amplification comprising two or more primers, and a detectable probe covalently attached to a fluorescent dye and a quencher moiety; wherein the detectable probe is covalently attached by a linkage to an aryl carbon of a quencher moiety selected from the diazo structures:





wherein Ar is C<sub>5</sub>-C<sub>14</sub> aryl; at least one aryl carbon of each diazo structure is substituted with an electron-withdrawing group and at least one aryl carbon of each diazo structure is substituted with an electron-donating group.

5

ADD  
B5

09942342

082781